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Structural characterization and *in vitro* inhibitory activities in P-selectin-mediated leukocyte adhesion of polysaccharide fractions isolated from the roots of *Physalis alkekengi*

Haibin Tong[a,b,c,1], Ruifei Wang[a,1], Ximing Liu[a], Guiyun Wang[a], Fengguo Du[c], Xianlu Zeng[a,*]

[a] *Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China*
[b] *Life Science Research Center, Beihua University, Jilin 132013, China*
[c] *College of Forestry, Beihua University, Jilin 132013, China*

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ABSTRACT

Selectin-mediated leukocyte initial attachment and rolling over vessel endothelial surface are crucial steps for inflammatory responses. As P-selectin is a promising target for anti-inflammation therapeutic strategy, recent works have focused on searching for more potent and non-toxic P-selectin antagonists among various natural carbohydrate products. Here, we isolated three water-soluble polysaccharide fractions (PPS-1, PPS-2 and PPS-3) from the roots of *Physalis alkekengi* by DEAE-cellulose and Sephacryl S-200 chromatography. Their physicochemical and structural characterizations were determined by chemical methods, GC (gas chromatography), HPLC (high performance liquid chromatography), FT-IR (Fourier transform infrared spectrometry), partial acid hydrolysis, methylation and GC–MS (gas chromatography–mass spectrometry) analyses. The inhibitory capacity of the polysaccharide fractions in P-selectin-mediated leukocyte adhesion was evaluated by flow cytometric, static adhesion and laminar flow assays. Results showed that different polysaccharide fractions possess distinct physicochemical and structural properties, including carbohydrate, protein and uronic acid contents, molecular weight, monosaccharide composition and glycosidic linkage type. Among the polysaccharide fractions, PPS-2 could effectively block the interaction between P-selectin and its native ligand.

1. Introduction

The recruitment of leukocytes from circulation to infected tissue is an important component of innate immune responses, which is a dynamic and coordinated multi-step process regulated by a variety of adhesion molecules and inflammatory factors [1,2]. Initial attachment of leukocytes with the vessel endothelial cells is termed tethering, and the subsequent rotational movement along the vessel endothelial surface is termed rolling. These events are mainly mediated by selectin family of adhesion molecule, which is carbohydrate-binding receptors on both endothelium and leukocytes [3,4].

P-selectin (CD62P) is constitutively expressed and stored in α-granules (in platelets) or Weibel–Palade bodies (in endothelium) [5]. When endothelium is stimulated by inflammatory mediators, the Weibel–Palade bodies rapidly fuse with the plasma membrane, which can present P-selectin on the cell surface and promotes the immediate attachment and rapid rolling of leukocytes over vascular surfaces. Therefore, P-selectin is an important adhesion molecule involved in early leukocyte recruitment during inflammatory response. Development of P-selectin antagonists has attracted much attention as a promising therapeutic strategy for inflammatory diseases [6].

Many kinds of carbohydrate compounds, including sLe^x oligosaccharides, sLe^x mimetics, diverse molecular weight of heparin, chitosan conjugate, fucoidin, mannan, natural polysaccharide and related polysaccharide conjugates have been examined in preclinical models and some clinical trials for their activities as competitive antagonist in inhibiting the interaction between P-selectin expressed on blood vessel endothelial cells and PSGL-1 (P-selectin glycoprotein ligand-1) expressed natively on circulating leukocytes [7–9]. However, the unfavorable pharmacokinetics, relatively low selectivity, low affinity, potential antigenicity, anticoagulation and relatively high production costs of these selectin antagonists made them unsuitable for further development into effective drugs for clinical application [10]. Among various natural products, polysaccharide extracted from medicinal herbs might prove to be one of

* Corresponding author at: Institute of Genetics and Cytology, School of Life Science, #5268, Remin Street, Changchun, Jilin 130024, China. Tel.: +86 431 85099317; fax: +86 431 85098735.
E-mail address: zengx779@nenu.edu.cn (X. Zeng).
[1] These authors contributed equally to this work.

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the promising candidates in searching for effective and non-toxic natural products with anti-inflammatory activity.

Physalis alkekengi belongs to the family of Solanaceae and is abundantly distributed in the northeast region of China, Korea and Japan. It is a traditional Chinese herbal plant, which has been officially listed in the Chinese Pharmacopoeia, with multiple pharmacological functions, such as anti-inflammatory, antioxidant, anti-fungal, hypoglycemic, febricide, expectorant and diuresis activities [11]. Though *P. alkekengi* has been reported to have anti-inflammatory effects, the mechanism remains unclear. In order to fully develop the wild resources and extend the potential use of *P. alkekengi*, this study was carried out to investigate the anti-inflammation mechanism of polysaccharide fractions from *P. alkekengi* roots, as well as their physicochemical and structural properties.

2. Materials and methods

2.1. Materials and chemicals

The roots of *P. alkekengi* were purchased from a local pharmaceutical market, and identified according to the identification standard of Pharmacopeia of the People's Republic of China.

Recombinant human P-selectin/Fc chimera protein (P–Fc) and blocking mAb to P-selectin (9E1) were obtained from R&D Systems (Minneapolis, Minnesota, USA). A non-blocking mAb to P-selectin (AC1.2) was purchased from BD PharMingen (Franklin Lakes, New Jersey, USA). Goat anti-human fluorescein-isothiocyanate (FITC)-labeled immunoglobulin G (IgG) and goat anti-mouse IgG were purchased from Jackson Immuno-Research Laboratories (West Grove, Pennsylvania, USA). Sephacryl S-200 and Superdex G-75 were purchased from Amersham Pharmacia Co. (Sweden). T-series dextrans were purchased from Fluka. DEAE-cellulose, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA) and standard monosaccharides were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trifluoroacetic acid (TFA), sodium hydroxide, inositol, acetic anhydride, pyridine, methanol and acetic acid were purchased from Beijing Chemicals and Reagents Co. (Beijing, China). All other chemical reagents used were analytical grade.

Human promyelocytic leukemia cells (HL-60) and CHO cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). CHO-P cells stably expressing human P-selectin were obtained by transfecting full-length human P-selectin vector into CHO cells, and then selected with G418. All cells were grown in IMDM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Isolation and purification of polysaccharide fractions

P. alkekengi roots were extracted with distilled water at 80 °C for three times and 3 h for each time. The whole extract was filtered and centrifuged. The supernatant was concentrated, and then precipitated with 3 volumes of ethanol. The crude polysaccharide precipitate was collected by centrifugation. Crude polysaccharide was deproteinated according to the method described by Tong et al. [12]. The deproteinated sample (cPPS) was dissolved in distilled water and loaded onto DEAE-cellulose column using an ÄKTA explore purification system, and eluted at a flow rate of 1 ml/min successively with distilled water and a gradient of 0→1 M NaCl. Individual fractions were collected by the automated fraction collector and monitored with the phenol–sulfuric acid method [13]. The individual fraction (PPS) was collected, dialyzed (*Mw* cut-off 3500 Da), lyophilized, and were further fractioned on a Sephacryl S-200 column (2.6 cm × 100 cm), eluted with 0.15 mol/L NaCl to yield three main fractions, codes as PPS-1, PPS-2 and PPS-3. All the collected fractions were dialyzed (*Mw* cut-off 3500 Da) and lyophilized to produce white purified polysaccharide powders.

2.3. Measurement of carbohydrate and protein contents

Total carbohydrate contents of the polysaccharide fractions were determined by phenol–sulfuric acid colorimetric method [13]. Total uronic acid contents were measured by *m*-hydroxydiphenyl method [14] using galacturonic acid as the standard. In addition, proteins in the polysaccharides were quantified according to the Bradford's method [15].

2.4. Molecular weight determination

Molecular weights of the different polysaccharide fractions were determined by high performance gel permeation chromatography [16]. The samples were dissolved in distilled water, loaded into a Shimadzu HPLC system equipped with a TSK-GEL G3000 PWXL column (7.8 mm × 300 mm), eluted with 0.1 mol/L Na_2SO_4 solution and detected by a RID-10A Refractive Index Detector (RID). Dextran standards with different molecular weights (T-2000, T-70, T-40, T-20 and T-10) were used to calibrate the column and establish a standard curve.

2.5. Analysis of monosaccharide composition

Polysaccharide fractions were hydrolyzed and acetylated according to the method of Lehrfeld [17]. The samples were hydrolyzed with TFA at 120 °C for 2 h. The hydrolyzed product was reduced with KBH_4, followed by neutralization with diluted acetic acid. Finally myo-inositol and Na_2CO_3 were added with stirring for 45 min. The residue was concentrated by adding methanol. Pyridine and propylamine were added into the reduced products (alditols) with stirring for 30 min at 55 °C, and acetylated with pyridine–acetic anhydride in a boiling water bath for 1 h. The acetylated products were analyzed by gas chromatography (GC), identified and estimated with myo-inositol as the internal standard.

GC was performed on a Varian 3400 instrument (Hewlett-Packard Component, USA) equipped with DM-2330 capillary column (30 m × 0.32 mm × 0.2 μm) and flame-ionization detector (FID). The column temperature was kept at 120 °C for 2 min, and increased to 250 °C (maintained for 3 min) at a rate of 8 °C/min. The injector and detector heater temperature were 250 °C and 300 °C, respectively. The rate of N_2 carrier gas was 1.2 ml/min.

2.6. Spectroscopic methods

Ultraviolet–visible spectra were recorded with a Varian Cary-100 Ultraviolet Visible Spectrophotometer (Varian Inc., Victoria, Australia). IR spectra were recorded with a Nicolet 5700 Fourier transform infrared spectrometer (Nicolet Instrument, Thermo Company, Madison, USA) in the range of 4000–400 cm^{-1}, using KBr-disk method.

2.7. Partial acid hydrolysis

Partial acid hydrolysis was performed as described by Cao et al. [18]. Polysaccharide fractions (PPS-1, PPS-2 and PPS-3 (50 mg)) were dissolved in 0.2 M TFA (5 ml) at 90 °C for 3 h, and then TFA was removed by addition of ethanol repeatedly. The hydrolyzed solutions were dialyzed (*Mw* cut-off 3500 Da) against distilled water for 48 h. Both dialyzable and non-dialyzable samples were lyophilized.